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Site-Specific Recombination-Based Genetic System for Reporting Transient or Low-Level Gene Expression

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Abstract

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Disciplines

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Comments

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Site-Specific Recombination-Based Genetic System for Reporting Transient or Low-Level Gene Expression†

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We report here the construction, characterization, and application of a plasmid-based genetic system that reports the expression of a target promoter by effecting an irreversible, heritable change in a bacterial cell. This system confers strong repression of the reporter gene *gfp* in the absence of target promoter expression and utilizes the site-specific recombination machinery of bacteriophage P22 to trigger high-level reporter gene expression in the original cell and its progeny after target gene induction. We demonstrate the effectiveness of this genetic system by tailoring it to indicate the availability of arabinose to the biological control agent *Enterobacter cloacae* JL1157 in culture and in the barley rhizosphere. The presence of bioavailable arabinose triggered the production of P22 excisionase and integrase from the reporter plasmid pAraLHB in JL1157, and this led to excision of the *c*I repressor gene, which is flanked by *att* sites, and the subsequent irreversible expression of *gfp* in the original cell and in its progeny. In culture, nearly 100% of an *E. cloacae* JL1157(pAraLHB) population expressed *gfp* after exposure to 6.5 to 65 μM arabinose for 3 h. We used this biosensor to demonstrate that arabinose was released from the seeds of several legumes and grass species during germination and from roots of barley seedlings grown hydroponically or in soil. When introduced into microcosms containing barley, the biosensor permitted the localization of arabinose along the roots. Arabinose was present near the root-seed junction and on the seminal roots but was not detected at the root tips. This recombination-based reporter system should be useful for monitoring bacterial exposure to transient or low levels of specific molecules directly in the environment.

Transcriptional fusions between a target gene and a reporter gene are a powerful tool for investigating bacterial gene expression in natural habitats and for detecting specific signal molecules in the environment (1, 5, 18, 23). Transcriptional fusions that are used for these purposes are often limited in their sensitivity, however, since low levels of reporter gene expression can be difficult to detect in cells from natural habitats, particularly if reporter gene expression is not assessed immediately after induction (23). Furthermore, such fusions do not effectively report transient expression, such as would result from encountering the inducer for only a limited period of time. The ability to detect whether a target gene was ever expressed, even at a low level, in a natural habitat would often be useful. For example, knowledge of the presence or absence of specific nutrients and environmental pollutants, both of which may be ephemeral or be present in only low concentrations, is important for understanding the ecology and bioremediation potential of an organism. For the same reasons, exposure of microbes to plant- or animal-derived compounds that induce specific microbial functions, including traits related to virulence, symbiosis, or biocontrol, may be difficult to detect. A genetic system that strongly represses the expression of a reporter gene when the target promoter is not being expressed and irreversibly induces a high level of reporter gene expression when the target promoter is expressed, even at low levels, would facilitate the detection of signal molecules in the natural environment.

To this end, we developed a genetic system that reports the expression of a target promoter by effecting an irreversible, heritable change in the biosensor cell; this system confers strong repression of a reporter gene in the absence of target promoter expression and high-level reporter gene expression in the original cell and its progeny after even a low level of target gene induction in the original cell. This system used the site-specific recombination elements of the *Escherichia coli* bacteriophage λ and *Salmonella enterica* serovar Typhimurium bacteriophage P22 and is related in concept to the recombination in vivo expression technology (RIVET) that has been used as a promoter trap to identify bacterial genes involved in virulence (4, 5, 21). As currently designed, RIVET requires that cells be reisolated from a habitat and screened for loss of an antibiotic resistance marker. The genetic system described in this report used the reporter gene *gfp*, encoding the green fluorescent protein (GFP) from *Aequoria victoria* (7). GFP is particularly useful as a reporter since, in addition to reporting gene activity based simply on inspecting cells or colonies for fluorescence, it permits visualization of the spatial patterns of gene expression directly in the habitat.

We used the rhizosphere as a model habitat to evaluate the efficacy of this recombination-based biosensor for detecting low amounts of a transiently available compound in a complex environment. The spermosphere and rhizosphere are nutrient-rich environments relative to the bulk soil (9). Seeds, as well as living and dying root tissue, release relatively labile sugars, amino acids, organic acids, and polysaccharides (16, 32, 33). The amount and composition of seed and root exudates are likely to differ depending on the age and physiology of the

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype and/or description[a]	Source or reference
Strains		
E. coli DH5α*pir*	*pir*$^+$; host strain for R6Kγ*ori* plasmids	30
E. coli DH5α	F$^-$ ϕ80d*lacZ*ΔM15Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17*(r_K^- m_K^+) *phoA supE44* λ$^-$ *thi-1 gyrA96 relA1*	Gibco-BRL
E. cloacae JL1157	Rf^r	J. Loper
Plasmids		
pRK2073	Sm^r; mobilization function	11
pGEM-T	Ap^r; cloning vector for PCR-amplified products	Promega
pPROBE-KT	Km^r; p15a and pVS1 *ori*; *gfp* promoter-probe vector	26
pBAD18	Ap^r; P_{BAD} promoter; *araC*	14
EMBL3	λ vector, source of P_L	12
pVP$_L$*gfp*	Km^r; pPROBE-KT containing λ P_L-*gfp*	This study
pXINTP22	Source of bacteriophage P22 *xis,int*	29
pBAD-*xis,int*	pBAD18 with a 1.4-kb *Eco*RI-*Sal*I *xis,int* fragment containing an RBS inserted distally to the P_{BAD} promoter	This study
pAra	Km^r; pVP$_L$*gfp* containing P_{BAD}-*xis,int*	This study
pT-*attP22*	Amp^r; pGEM-T vector containing *attP22*	This study
pCDP22SK	Cm^r; R6Kγ*ori; attP*	29
pINTKanP22	Km^r; P22 *int*; helper plasmid for integration into *attP22* sites; contains temperature-sensitive *ori*	G. Phillips
pCDP22SK-*c*I	pCD22PSK with λ *c*I repressor gene	29
pT-*att*-*c*I	pT-*att*-CD-R6K with R6Kγ*ori* excised; contains the λ*c*I cassette (*attL*-Cm^r-*c*I-*attR*)	This study
pAraLHB	pPROBE-KT containing P_L-*gfp*, P_{BAD}-*xis,int*, and λ *c*I cassette from pT-*att*-*c*I	This study
pVLacRed	P_{lac}-DsRed	C. Axtell

[a] Sm^r, streptomycin resistance; Ap^r, ampicillin resistance; Rf^r, rifampin resistance; Ap^r, ampicillin resistance; Km^r, kanamycin resistance; Cm^r, chloramphenicol resistance. RBS, ribosome-binding site.

plant and on the sites that are examined on the root (16), particularly relative to the site of seed germination. Thus, specific components in these exudates may be produced only transiently or may be used by the colonizing microflora at such a rapid rate that their availability is only transient or there are only low levels available. Because the temporal and spatial distribution of specific nutrients may be complex, identifying whether an organism has access to a specific nutrient in the rhizosphere is challenging. The ability of a microorganism to access and utilize specific nutrients, however, may be critical to both its fitness in the rhizosphere and its relative competitiveness within the rhizosphere community.

We developed a bacterial sensor to detect available arabinose by using the biocontrol agent *Enterobacter cloacae* JL1157 (8, 24). Arabinose is a component of seedling exudates (31) and is a constituent of many cell wall polymers of grasses and thus may be present in root-derived compounds through exudation, secretion, or, indirectly, plant cell wall damage (6). It is likely to be one of the many carbohydrates that can be used by *E. cloacae* during spermosphere and rhizosphere colonization. We constructed an arabinose-inducible, recombination-based bacterial biosensor by using the *E. coli* L-arabinose-inducible *araBAD* promoter (P_{BAD}), the associated AraC regulator (14, 19, 27), and various site-specific recombination elements from bacteriophage λ and P22. We describe here the construction of this system and demonstrate its use in detecting the availability of arabinose to *E. cloacae* in the barley rhizosphere.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this study are described in Table 1. *E. coli* and *E. cloacae* were routinely cultivated on Luria agar or Trypticase soy agar (TSA), respectively. Media were supplemented with antibiotics when appropriate at the following concentrations: ampicillin, 50 μg/ml; chloramphenicol, 50 μg/ml; kanamycin, 50 μg/ml; and rifampin, 20 μg/ml. For the arabinose dose-response curves and inhibitor studies, *E. cloacae* was grown in a semidefined medium, yeast extract (YE)-succinate broth or YE-glycerol broth containing 0.5 g of NH_4Cl, 1.75 g of $Na_2HPO_4 \cdot 7H_2O$, 1.38 g of KH_2PO_4, 0.2 g of YE, and 40 ml of Huntner mineral solution (37)/liter and either 7 mM succinate or 26 mM glycerol, and the pH was adjusted to 6.8. Solid media contained 15 g of agar/liter.

Genetic techniques. Standard methods were used for DNA isolation, restriction digests, ligations, and transformation (34). Restriction site modifications were performed by treating 10 pmol of oligonucleotide adapters with T4 kinase, heating the mixtures to 80°C, and then slowly cooling them to room temperature prior to ligation. PCR amplification targets, primers, and amplification conditions are described in Table 2. Plasmids were mobilized into *E. cloacae* by triparental mating using the mobilization function of plasmid pRK2073 (11).

Biosensor construction. The components of the pAraLHB biosensor were assembled into pPROBE-KT (26), a broad-host-range promoter probe vector, by using amplified products as the source of key elements (see Table 2 for a description of the PCR protocols) and standard recombinant DNA techniques. The strategy employed in pAraLHB construction is shown in Fig. 1. First, we generated a fusion between the arabinose-responsive promoter P_{BAD} and a promoterless copy of the P22 operon containing the integrase (*int*) and excisionase (*xis*) genes (Fig. 1A). This was accomplished by amplifying the *xis,int* operon from pXINTP22 (29), which included a ribosomal binding site, and cloning it into the *Eco*RI-*Sal*I site of pBAD18 (14) to generate pBAD-*xis,int*. Second, the bacteriophage λ promoter P_L, including the operator sites, was amplified from λ EMBL3 (Table 2) and cloned into the *Bam*HI site of pPROBE-KT to generate pVP$_L$*gfp* (not shown). Third, we amplified a 3.2-kb portion of pBAD-*xis,int* that included the transcriptional activator *araC*, the P_{BAD}-*xis,int* fusion, and the *rrnB* transcriptional terminators, and cloned this fragment into the *Kpn*I site of pVP$_L$*gfp* to create pAra (Fig. 1B). Fourth, we generated a cassette in which the λ *c*I repressor gene was flanked by attachment (*att*) sequences that are recognized by bacteriophage P22 excisionase and integrase proteins (Xis and Int, respectively) (Fig. 1C). This was accomplished by amplifying *attP22*, which is recognized by the bacteriophage P22 integrase, and introducing it into pGEM-T to form pT-*attP22*. Cointegration of pT-*attP22* and plasmid pCDP22SK, which contained *attP*, was facilitated by pINTKanP22 by the technique described by Platt et al. (30). Plasmid pINTKanP22 mediated cointegrate formation via its constitutive production of integrase, and it was lost via growth at a nonpermissive temperature (42°C). The 1.1-kb *Xba*I fragment containing the R6Kγ*ori* in the

TABLE 2. PCR protocols

Gene and/or description	Template	Primer		Amplification conditions[b] (reagent)
		Name	Sequence[a]	
P_L	λ EMBL3	5′pl	TGCCTCACGATCGCC	3 cycles of 94°C for 45 s, 47°C for 45 s, and 72°C for 1 min, followed by 19 cycles of 94°C for 45 s, 60°C for 45 s, and 72°C for 1 min (Amplitaq)
		3′pl	GCTGATGTGCTCAGTATCAC	
xis,int	pXINTP22	RBSXis	AGGAGTAAACATGGAATC	3 cycles of 94°C for 45 s, 48°C for 45 s, and 68°C for 1.5 min, followed by 22 cycles of 94°C for 45 s, 55°C for 45 s, and 68°C for 1.5 min (ExTaq)
		3′Int	ACTTACGTATTATTCGTGCC	
araC-P_{BAD}-*xis,int* including *rmB* terminator sites	pBAD-*xis,int*	araC	TGGACGAAGCAGGGATTC	6 cycles of 94°C for 45 s, 48°C for 45 s, and 68°C for 3 min, followed by 20 cycles of 94°C for 45 s, 55°C for 45 s, and 68°C for 3 min (ExTaq)
		bla-end	AGGGCGACACGGAAATG	
attP22 site	DH5α genome	att1	GGCACAACACTCCGAT	25 cycles of 94°C for 45 s, 50°C for 45 s, and 72°C for 45 s (Amplitaq)
		att2	GCCAAGGATGTATAGTGAGCGAA	

[a] The reported sequences do not include restriction sites, which were added when necessary. When restriction sites were added, oligonucleotides included at least six additional bases for enzyme recognition sites, as well as two to four additional nucleotides at the 5′ end. Amplified products were ligated into pGEM-T.
[b] Each amplification was started with an initial denaturation step of 94°C for 2 min and ended with a final 6-min extension at 72°C.

pT-*att*-CD-R6K cointegrate was replaced with a 1.2-kb *Eco*RV-*Xba*I fragment containing the λ *c*I repressor gene from pCDP22SK-*c*I (29) to create pT-*att*-*c*I. Fifth, a 4.5-kb *Cla*I fragment containing the λ *c*I cassette was excised from pT-*att*-*c*I and was inserted into the *Cla*I site of pAra to generate pAraLHB (Fig. 1D).

In vitro testing of the biosensor. *E. cloacae* JL1157(pAraLHB) was cultivated on TSA supplemented with antibiotics. Exponential-phase cultures were prepared by inoculating single colonies into fresh YE-succinate broth and incubating the cultures for 1 to 2 h at 28°C in an orbital shaking incubator set at 200 rpm prior to the addition of arabinose. The cultures were exposed to 6.6×10^{-7} to 6.6×10^{-3} M arabinose for various periods of time (1, 3, or 6 h), and subsamples were serially diluted in phosphate-buffered saline prior to plating onto TSA-kanamycin by using a spiral plater (Spiral Biotech, Bethesda, Md.). Plates were incubated at 28°C for 1 to 2 days, the colonies were enumerated, and the proportion of the colonies producing GFP was determined visually by using a UV handheld lamp (λ_{ex} = 365 nm) or a Dark Reader handheld lamp (λ_{ex} = 420 to 500 nm; Clare Chemical Research, Denver, Colo.) and filtered glasses that collected emission wavelengths of >520 nm, which facilitated the visual detection of green fluorescence. The same procedure was used to evaluate the responsiveness of stationary-phase JL1157(pAraLHB) biosensor cells to arabinose except that the cells were cultivated for 24 h prior to the addition of arabinose.

In situ application of the biosensor. Clarion soil (Typic Hapludoll; fine-loamy, mixed mesic) was sieved through a 2-mm mesh and stored at 4°C until use. JL1157(pAraLHB) was grown overnight on 1/5-strength TSA amended with kanamycin and rifampin; a single, nonglowing colony was restreaked, and the cells were resuspended in phosphate-buffered saline for inoculation of either soil or roots. The soil microcosm (no plants) consisted of 30 g of soil in a 50-ml screw-cap test tube that was gently packed to generate a bulk density of ca. 1.6 g/cm^2. In the soil-only experiments, an aliquot of JL1157(pAraLHB) was added directly to the soil to achieve ca. 10^7 to 10^8 CFU/g of soil with a sufficient inoculum volume to achieve a final soil water content of 15% (wt/wt). Preliminary experiments showed that *E. cloacae* maintained population sizes of between 10^6 to 10^7 CFU/g in nonrhizosphere soil over a 14-day period. For the rhizosphere experiments, barley seeds (*Hordeum vulgare* cv. Manchurian) were surface disinfested by soaking them in 0.5% sodium hypochlorite (vol/vol) for 20 min, were rinsed extensively with sterile water, and were germinated in the dark for 48 h on moistened filter paper. Barley seedlings were dipped in 10^{10} CFU of JL1157(pAraLHB)/ml cell suspensions and were grown in soil microcosms (600 g of soil in Styrofoam cups), in rhizotrons for microscopy, or in a hydroponic system. The rhizotrons for microscopy consisted of microscope slides placed over a 3-mm spacer, and the chamber was filled with soil. The edges of the chambers were sealed with masking tape. Rhizotrons were placed at a 45° angle to ensure that the roots grew along the surface of the microscope slide. The hydroponic system consisted of wrapping the seedling in sterile Whatman filter paper and placing it in a flask of half-strength plant nutrient solution (3). Plants were grown in a growth chamber set at 21°C and 99% humidity with a 12-h (light)–12-h (dark) photoperiod for 3 to 5 days.

Microscopy. Samples were viewed on a Nikon EFD-3 epifluorescence microscope, and images were taken with a charge-coupled-device camera (SPOT Camera; Diagnostic Instruments, Sterling Heights, Mich.). GFP fluorescence was imaged by using a filter set with an excitation wavelength of 480 ± 15 nm and an emission wavelength of 535 ± 20 nm. DsRed fluorescence was imaged by using a (470 ± 20)-nm excitation and a 515-nm long-pass emission filter set. Images were cropped by using Adobe Photoshop (Adobe Systems, Mountain View, Calif.).

Statistical analyses. Statistical analyses were performed by using JMP version 4.04 (SAS Institute, Cary, N.C.). Analysis of variance for comparing the proportion of JL1157(pAraLHB) cells that expressed *gfp* at different incubation times was performed on the arcsine square-root transformation of the proportion of cells that expressed *gfp*. The Tukey-Kramer honestly significant difference (HSD) means test (P = 0.01) was calculated by JMP software for comparisons among treatment means.

RESULTS

Construction of the biosensor system. Plasmid pAraLHB (Fig. 1) was derived by inserting genes or genetic elements into the pPROBE-KT (26) vector as outlined in Materials and Methods and shown in Fig. 1. The phage λ promoter P_L, which was inserted into pPROBE-KT to form a P_L-*gfp* fusion, included the operator sites O_{L1}, O_{L2}, and O_{L3} that bind the λ *c*I repressor. The arabinose-inducible promoter P_{BAD} of *E. coli* was fused ca. 25 bases from the ribosomal binding site of a promoterless copy of the bacteriophage P22 *xis,int* operon. The gene encoding the regulator of P_{BAD}, *araC*, was expressed constitutively under the control of its cognate promoter. In the resulting biosensor strain JL1157(pAraLHB), arabinose-mediated induction of the P_{BAD}-*xis,int* transcriptional fusion led to

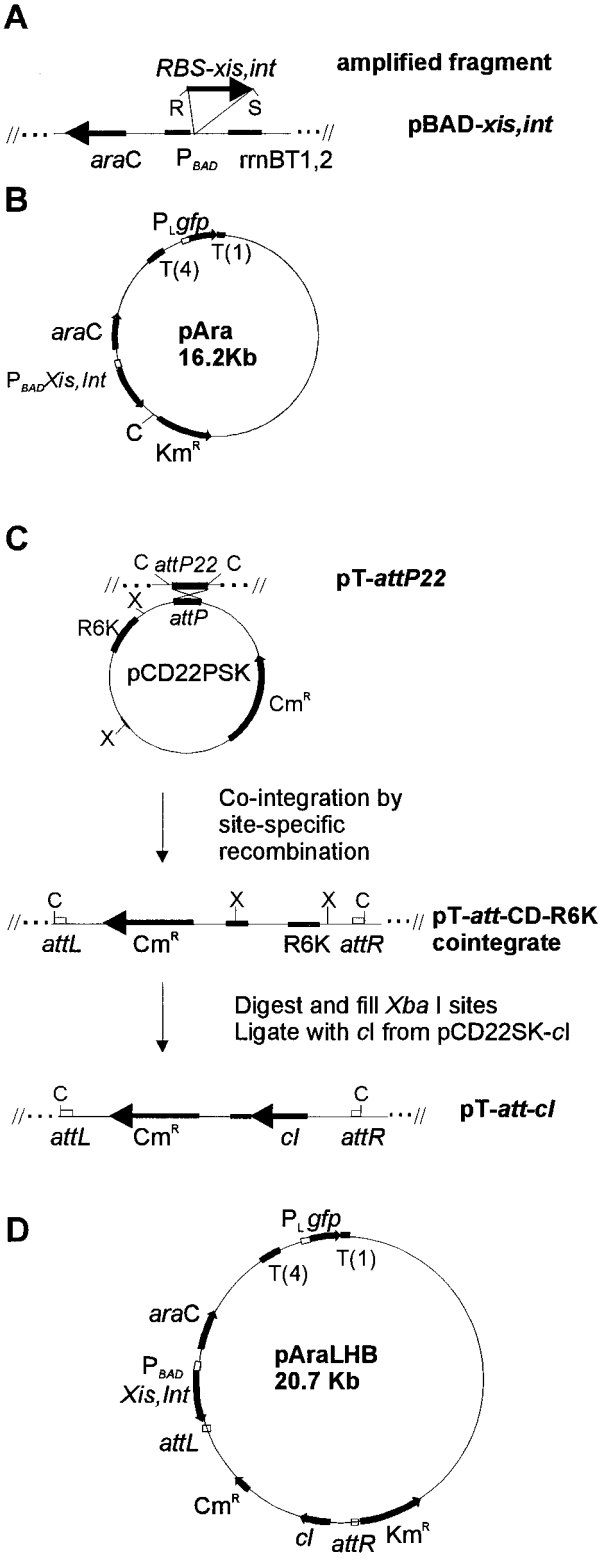

FIG. 1. Schematic illustration of the arabinose life history biosensor (pAraLHB) construction. (A) Construction of P_{BAD}-*xis,int* fusion. The amplified 1.4-kb fragment containing *xis,int* (Table 2) was ligated into the *Eco*RI and *Sal*I sites of pBAD18 adjacent to the P_{BAD} promoter. (B) Diagram of pAra. A 0.2-kb amplification product (Table 2) containing the λ promoter P_L was inserted into the *Bam*I site of pPROBE-KT. A 3.2-kb amplification product (Table 2) containing *araC* and P_{BAD}-*xis,int* and an *rrnB* terminator, which was generated by using pBAD-*xis,int* as a template, was inserted into the *Kpn*I site of pPROBE-KT. (C) Construction of the λ *c*I cassette. The *attP22* and *attP* sites were obtained from pT-*attP22* and pCDP22SK, respectively, to generate the pT-*att*-CD-R6K cointegrate by using P22 integrase. pT-*att*-*c*I was derived by deleting a 1.1-kb *Xba*I fragment that included the R6Kγ*ori*, filling in the *Xba*I sites, and inserting a 1.2-kb fragment containing the λ *c*I gene from pCDP22SK-*c*I. (D) Diagram of pAraLHB. A 4.2-kb *Cla*I fragment from pT-*att*-*c*I, which contained the *attL*-*c*I-Cm^r-*attR* cassette, was inserted into the *Cla*I site of pPROBE-KT. Abbreviations: RBS, ribosomal binding site; R, *Eco*RI; S, *Sal*I; C, *Cla*I; X, *Xba*I; Cm^R, chloramphenicol resistance gene; R6K, R6Kγ origin of replication; T(1) and T(4), *E. coli rrnB* T1 terminator(s); rrnBT1,2, *E. coli rrnB* T1 and T2 terminators.

Xis- and Int-mediated site-specific recombination at the *att* sequences, causing excision of the *c*I cassette. Since the *c*I cassette does not contain an origin of replication, it is subsequently lost from the cell.

Arabinose induction of *E. cloacae* JL1157(pAraLHB) in vitro. Throughout these studies, the proportion of cells in a population that was exposed to sufficient levels of arabinose to induce P_{BAD}-*xis,int* expression was determined based on the proportion of fluorescent colonies that arose after plating a sample of the population. This assay for P_{BAD} induction, and thus arabinose availability, was possible due to the irreversible genetic change that occurred in the induced cells. In preliminary studies, we observed that a single chromosomal copy of the λ *c*I gene blocked P_L-mediated *gfp* expression from a pVS1-based plasmid containing the P_L-*gfp* fusion (data not shown). Therefore, for high-level *gfp* expression to occur, sufficient quantities of both Xis and Int proteins must be produced to remove the λ *c*I cassette from all copies of the vector.

The P_L promoter was not sufficiently leaky to cause visible green fluorescence in colonies of uninduced cells. During growth in a nutrient rich medium (e.g., Luria broth and YE-glycerol) in the absence of exogenous arabinose, excision of the *c*I cassette occurred in only 0.5% of the population per generation, which generally resulted in excision of the *c*I cassette in 1 to 13% of a culture population based on colony fluorescence. The leakiness from the P_{BAD} promoter was reduced by a carbon source that functions as a catabolite repressor of the P_{BAD} promoter (14, 27). We found that this leakiness was, in fact, reduced by cultivating cells with glucose, as well as with chloramphenicol, which selected for cells that had not lost the *c*I cassette (data not shown). A chloramphenicol- and glucose-containing nutrient rich medium (i.e., TSA) was thus used to prepare inocula that contained a minimum number of cells that had lost the *c*I cassette and were irreversibly green fluorescent. We also determined that *gfp* expression in the fluorescent colonies after arabinose exposure was due to removal of the *c*I cassette since 100% of the colonies examined were chloramphenicol sensitive. Furthermore, we determined that cells obtained from non-green fluorescent colonies that had previously been exposed to arabinose exhibited loss of the *c*I cassette after subsequent arabinose exposure, which indicates that the failure of a cell to lose the *c*I cassette upon arabinose exposure was not due to a genetic change that prevented excision of the *c*I cassette. Doubling times (mean ± the standard

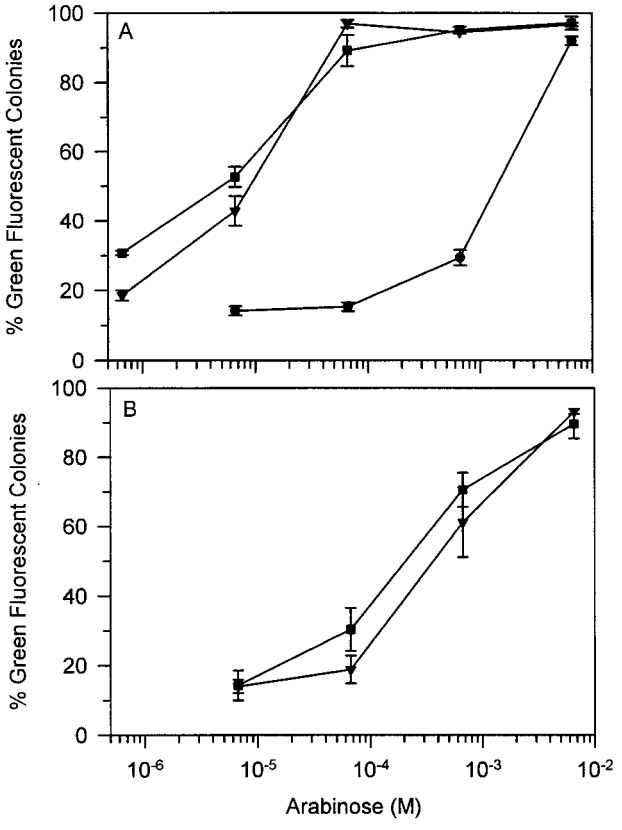

FIG. 2. Dose response of JL1157(pAraLHB) in YE-succinate broth amended with various concentrations of arabinose and incubated for 1, 3, or 6 h. (A) Exponential-phase cultures. (B) Stationary-phase cultures. The symbols represent the duration of arabinose exposure as follows: ●, 1 h; ▼, 3 h; ■, 6 h. All points represent the mean of three replicates, and the error bars represent the SEM. The background green fluorescent cells in the exponential- and stationary-phase populations were 13.4% ± 4.3% and 6.8% ± 1.2%, respectively (mean ± the SEM).

error of mean [SEM]; $n = 3$) of JL1157(pAraLHB) cultures derived from cells that constitutively expressed *gfp* (42.5 ± 1.4 min) were comparable to those of JL1157(pAraLHB) cultures that did not express *gfp* (45.4 ± 1.0 min), indicating that high-level *gfp* expression did not impose a fitness cost during growth in Trypticase soy broth (TSB), and similar results were obtained when YE-succinate was the growth medium (data not shown).

To determine the rapidity with which site-specific recombination took place upon exposure to arabinose, we examined the kinetics of the acquisition of green fluorescence in cultures exposed to various concentrations of arabinose in YE-succinate broth. After exposure to 65 μM arabinose, nearly 100% of the colonies exhibited green fluorescence after 3 h, and a significant subpopulation (40 to 50%) of cells was fluorescent after exposure to only 6.5 μM arabinose for 3 h (Fig. 2A). Shorter incubation times required higher arabinose concentrations to achieve 100% of the population expressing GFP. Similar results were obtained in replicate experiments where the carbon source was glycerol (i.e., YE-glycerol; data not shown). In experiments with stationary-phase JL1157(pAraLHB) cells, a higher arabinose concentration and a longer incubation period were required for site-specific recombination to take place (Fig. 2B). The requirement for higher arabinose concentrations and prolonged incubation periods (≥3 h) indicates that the cell's physiological state affects its ability to respond to arabinose. This result suggests that a low-energy state, such as occurs in stationary cells, increases the threshold concentration and reduces the rate at which the arabinose-induced genetic change occurs in JL1157(pAraLHB) cells.

Arabinose induction of JL1157(pAraLHB) by plant exudates or in planta. In the 2-week period after introduction of JL1157(pAraLHB) into soil microcosms, a similar percentage of fluorescent cells was recovered as in the original inoculum (12% ± 3.3% for each [mean ± SEM]; $n = 3$) ($P < 0.05$). This percentage increased to 73 to 86% of the cells exhibiting green fluorescence after 24 h of exposure to 2 mM arabinose, whereas only 12.4% ± 3.3% exhibited fluorescence in the unamended controls. JL1157(pAraLHB) residing in soil for up to 14 days after inoculation before arabinose amendment responded to arabinose by exhibiting a similar percentage of fluorescent cells (83% ± 7.5%) as a population that had been residing in soil for only 1 day before arabinose amendment (74% ± 3.5%).

We examined various seed exudates for the presence of arabinose by incubating JL1157(pAraLHB) for 6 h in a 1:1 YE-glycerol–seed exudate mixture before the exudates were plated to determine the proportion of the population that exhibited fluorescence. The proportions of *E. cloacae* cells that expressed *gfp* in the presence of seed exudates from the barley, maize, soybean, radish, cucumber, alfalfa, and the negative control treatments were 42.5% 177± 7.5%, 42.8% ± 1.1%, 44.7% ± 2.3%, 23.6% ± 1.1%, 35.2% ± 7.6%, 20.6% ± 1.5%, and 2.7% ± 0.8% (mean ± standard deviation; $n = 2$), respectively. Although it is possible that other sugars, such as glucose, which is a known component of many seed exudates (31), inhibited arabinose-mediated induction in some of the cells, the significant increase in the percentage of *gfp*-expressing cells in the presence of each of the seed exudates relative to the negative control provides strong evidence that arabinose was present in each of the seed exudates tested.

We examined the availability of arabinose in the barley rhizosphere by dipping 30- to 40-h-old barley seedlings in a JL1157(pAraLHB) cell suspension prior to planting them in a hydroponic system. The background population of JL1157(pAraLHB) cells that expressed *gfp* was determined by immediately plating excised roots that had been dipped in the cell suspension (Fig. 3). At the time of inoculation (day 0) only 2.1% ± 0.1% of the population expressed *gfp*, but within 24 h ca. 50% of the population expressed *gfp* (Fig. 3). There was no statistical difference in the proportion of JL1157(pAraLHB) cells that expressed *gfp* among the different sampling times based on a Tukey-Kramer HSD test ($P = 0.01$), except between days 3 and 4. It is possible that the decrease between days 3 and 4 resulted from population decreases in regions containing arabinose with simultaneous population increases in areas devoid of arabinose. The population sizes of JL1157(pAraLHB) in the barley rhizosphere of hydroponically grown plants 1 to 4 days after inoculation did not change significantly over time (Fig. 3). In a parallel experiment with plants grown in soil, we determined that the percentage of

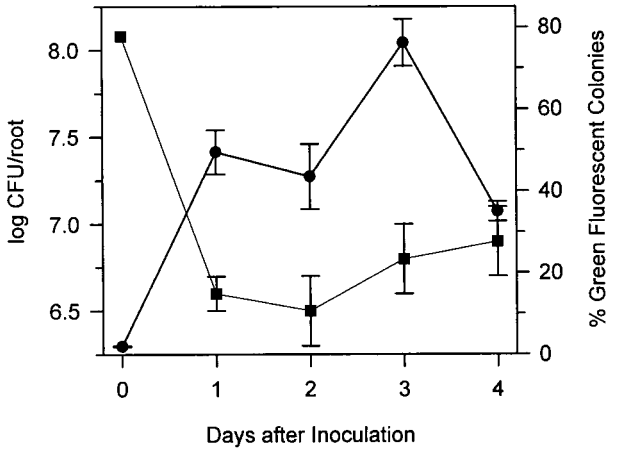

FIG. 3. Detection of available arabinose in the barley rhizosphere of hydroponically grown plants with the JL1157(pAraLHB) biosensor. Symbols: ●, percentage of JL1157(pAraLHB) cells that expressed *gfp*; ■, $\log_{10}$ CFU/root. Points represent the mean ± the SEM of four replications per sampling time.

rhizosphere JL1157(pAraLHB) cells that expressed *gfp* increased from 1.3% ± 0.2% immediately after inoculation to 56.5% ± 14.6% 5 days after inoculation (mean ± SEM; $n = 3$). The higher variability in the soil-grown plants was likely a consequence of including in the rhizosphere samples soil particles that contained uninduced JL1157(pAraLHB) cells. The population sizes of JL1157(pAraLHB) in the barley rhizosphere 1 to 5 days after inoculation were typically 6.5 ± 0.2 $\log_{10}$ (CFU/root) for the soil-grown plants.

We also examined the spatial distribution of available arabinose around barley roots by introducing a mixture of JL1157(pAraLHB) and JL1157(pVLacRed) cells onto plants either by inoculating soil with a cell suspension prior to planting a 2-day-old seedling or by dipping a barley seedling in a cell suspension prior to planting. Strain JL1157(pVLacRed), which constitutively expressed the red fluorescent protein DsRed, was included to facilitate visualizing the distribution of the inoculated bacteria. Green fluorescent bacteria were rarely seen in the soil; in contrast, red fluorescent bacteria were distributed throughout the soil (Fig. 4A). Green fluorescent bacteria were frequently observed on the rhizoplane of the seminal (primary) roots (Fig. 4B, C, and D), less frequently on or near the root hairs of any root (Fig. 4C), and rarely on the adventitious (secondary) roots. The greatest number of green fluorescent cells and colonies were located on the seminal roots near the seed-root junction (Fig. 4B and C). Green fluorescent bacteria seldom covered large areas, nor were they usually associated with root tips. Given the abundance of the red fluorescent cells on the roots and in the soil, indicating widespread distribution of the introduced bacterial cells, the abundance of the green fluorescent cells on the roots compared to the occasional green fluorescent cell in the soil indicates that arabinose was available near the root but was limited, or absent, in the soil. Whether the origin of the arabinose was the seed or the seed and the roots could not be determined.

DISCUSSION

This report describes the construction of a genetic system that effectively reports the presence or absence of gene expression in response to transient and/or low levels of an inducing signal. In this system, the production of a site-specific recombinase is controlled by the promoter of a gene of interest, if gene expression itself is being studied, or a target signal-dependent promoter, if the presence of a target signal is being studied. Expression of this promoter, even at low levels, induces a site-specific recombination event, and this recombination results in the permanent and heritable loss of a repressor gene that is necessary for blocking expression of the reporter gene, *gfp*. This system consists of four components: (i) a fusion between the promoter of interest and a promoterless copy of the bacteriophage P22 operon containing the excisionase and integrase genes; (ii) a fusion between the bacteriophage λ promoter P_L and a promoterless reporter gene; (iii) constitutive expression of any transcriptional activators that are needed for expression of the promoter of interest; and (iv) a λ *c*I repressor gene that is flanked by P22 *att* sites, which are the DNA sequences at which the enzymes excisionase and integrase function. In the absence of expression from the promoter of interest, the λ *c*I repressor tightly represses the expression of the P_L-reporter gene fusion. After induction of the promoter of interest, the enzymes excisionase and integrase are produced; they recognize the *att* sites and catalyze a recombination event that excises the *c*I repressor gene. Loss of the *c*I repressor protein results in irreversible expression of the P_L-reporter gene fusion, which is relatively highly expressed in the organisms tested. This entire genetic system was placed on a stable, multicopy, broad-host-range vector (26), which allows it to be readily introduced into other bacteria.

We demonstrated the effectiveness of this genetic system by tailoring it to indicate the availability of arabinose to the biocontrol agent *E. cloacae* JL1157 in culture and in the barley rhizosphere (Fig. 2 to 4). Arabinose is one of many different carbohydrates available to bacteria in the spermosphere and/or rhizosphere, and it is not in great abundance relative to other carbohydrates (6, 31). Specifically, we examined the expression of the *E. coli* L-arabinose-inducible *araBAD* promoter (P_{BAD}), which is one of the best-characterized promoter regulatory systems. The binding of arabinose to the AraC regulatory protein induces the expression of the *ara* loci, and the resulting production of arabinose transporters results in higher internal arabinose concentrations and hence further induction. This autocatalytic mechanism of induction results in all-or-none expression of the P_{BAD} promoter (35). That is, cells exposed to arabinose concentrations below a threshold exhibit no P_{BAD} activity, whereas those exposed to concentrations above a threshold exhibit P_{BAD} activity at a level that is not influenced by arabinose concentration. The threshold arabinose concentration may not be identical for all of the cells in a population, however. Thus, a population exposed to an intermediate arabinose concentration is likely to contain both P_{BAD}-expressing and P_{BAD}-nonexpressing cells. Previous studies demonstrated that the proportion of a population that exhibits P_{BAD} activity is influenced by arabinose concentration within the range of 1.33 to 133 μM (14, 19, 35). Similarly, we demonstrated that the proportion of a population that exhibited P_{BAD}-mediated

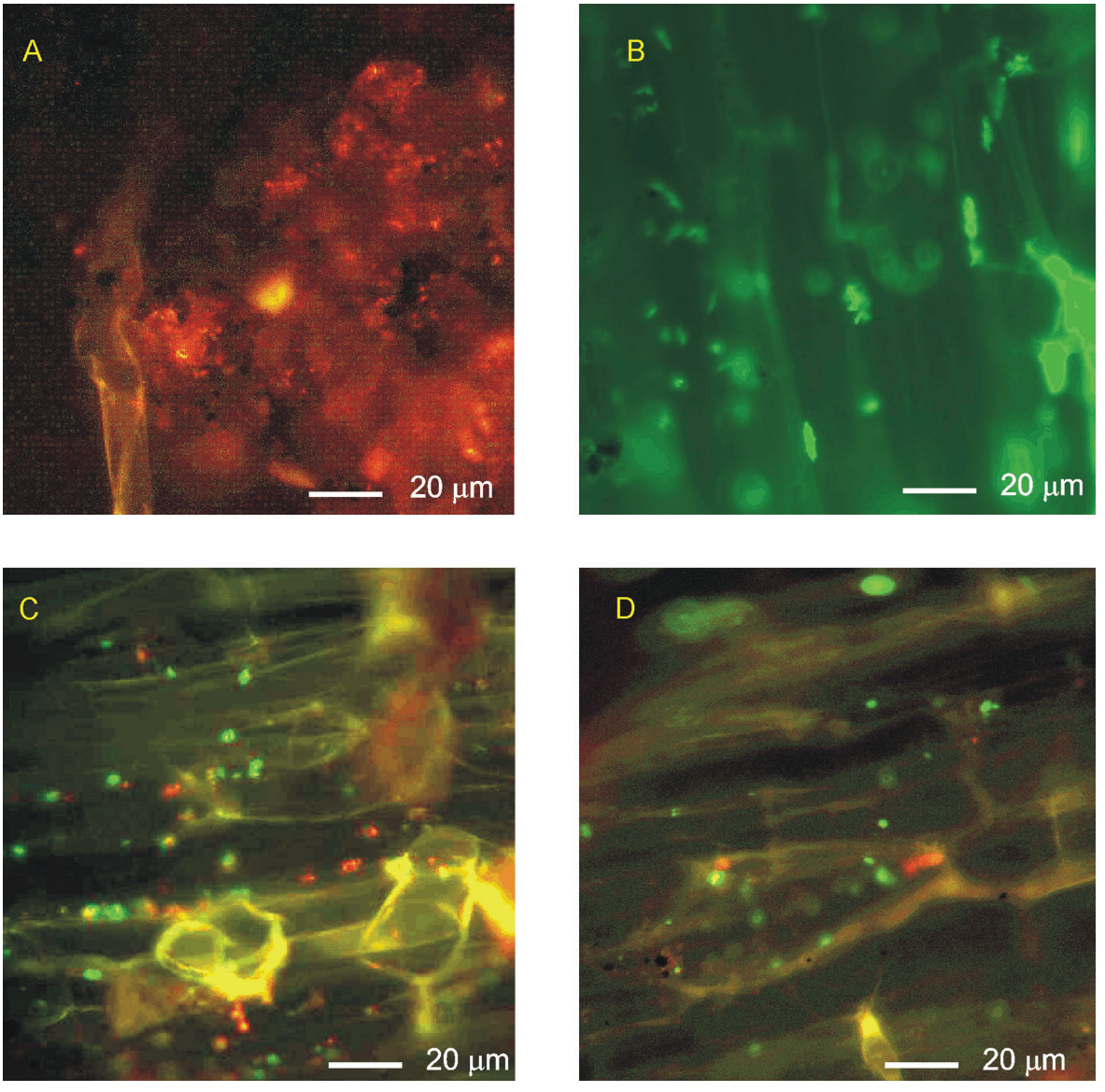

FIG. 4. Detection of available arabinose in the rhizosphere of barley growing in nonsterile soil with the JL1157(pAraLHB) biosensor. All panels show roots in microcosms inoculated with a mixture of JL1157(pAraLHB) (green fluorescence) and JL1157(pVLacRed) (constitutive red fluorescence), except for the roots in panel B, which were inoculated only with JL1157(pAraLHB). Green fluorescence indicates the presence of arabinose. Bars, 20 μm. (A) Red fluorescent bacteria in soil adjacent to a root hair. No green fluorescent bacteria were visible in the soil or along the root hair. (B) Green fluorescent bacteria forming microcolonies on the seminal root near the seed-root junction (the image obtained was taken 4 days after inoculation). (C) Red and green fluorescent bacteria on a seminal root close to the seed-root junction. (D) Red and green fluorescent bacteria on the same root as shown in panel C, but farther down the root axis.

site-specific recombination was influenced by arabinose concentration. Specifically, only 52% of the cells had experienced recombination after exposure to 6.5 μM arabinose (Fig. 2), whereas almost 100% of the population had undergone recombination after exposure to 65 μM arabinose. This suggests that in metabolically active cells, the dominant factor affecting the removal of the *c*I cassette is the kinetics of P_{BAD}-*xis,int* expression rather than the kinetics of Xis and Int protein production or Xis- and Int-mediated recombination.

The absence of an increase in the percentage of fluorescent biosensor cells after their introduction into soil could indicate that arabinose was not readily available in the soil but more likely reflects the entry of the JL1157(pAraLHB) cells into a physiological state in which the cells did not exhibit a visible response to arabinose. The latter possibility is supported by the fact that soils are generally viewed as nutritionally limited, and stationary-phase cells, which are nutritionally limited, also exhibited a small visible response to arabinose. In fact, it is possible that in both the stationary-phase culture and the soil populations examined, the cells that exhibited fluorescence after exposure to arabinose did so only after the arabinose was utilized as a carbon source, thus changing their physiological status to one with greater energy reserves. This would explain why the stationary-phase cultures and soil populations exhib-

ited a much longer period between arabinose exposure and the appearance of fluorescent cells than did exponential-phase cultures. Among the steps required for the cells to exhibit a visible response to arabinose, the arabinose transport step and the Xis- and Int-mediated recombination step are prime targets for inhibition in a nutrient-limited cell because of the high energy requirements of both permease synthesis and recombination (38). Since the in vivo half-life of excisionase (ca. 4 to 7 min) is considerably shorter than that for integrase (ca. 60 min) in wild-type *E. coli* cells, recombination will be particularly dependent on excisionase levels (22, 39). In the genetic system described here, the excisionase levels depend on the strength of the signal-responsive promoter that controls *xis*/*int* expression, as well as on physiological factors that influence target promoter expression or excisionase stability. Previous studies have demonstrated that the Lon and FtsH proteases target degradation of excisionase (22); thus, any factors that influence the activity of these proteases, such as starvation and heat shock, will also influence recombination.

The results of the soil study illustrate the importance of using this recombination-based biosensor system for the study of metabolically active cells. Although the poorer response in stationary-phase cells versus exponential-phase cells may be unique to this arabinose biosensor, it is likely that the effect of a cell's metabolic status on the ability to respond to a signal is an important factor to consider with any recombination-based reporter system. Alternatively, the poorer response in stationary-phase cells may be a consequence of the need for cell division to dilute the concentration of *c*I repressor sufficiently so that the P_L-*gfp* fusion would be derepressed. Previous studies that employed recombination-based genetic tools have not documented this phenomenon.

The proportion of JL1157(pAraLHB) cells that was fluorescent was significantly larger for populations recovered from the rhizosphere of barley seedlings than for cells in the original inoculum, providing strong evidence that arabinose was available to the rhizosphere microflora under these conditions (Fig. 3). Use of the JL1157(pAraLHB) biosensor in culture with seed exudates provided clear evidence that barley seed exudates contained arabinose and that the sugar was available mostly in regions near the seed-root junction (Fig. 4B and C). Although the arabinose on the roots may have originated in the seed and arrived at the roots during leakage of the seed exudate, the arabinose on the roots may also have originated in the roots. Arabinose is a component of various cell wall polymers of the grasses, including glucuronoarabinoxylans, arabinans, and arabinogalactans (6). In dividing and elongating cells, such as in young roots, glucuronoarabinoxylans are highly branched with arabinosyl units; these arabinosyl units are not cleaved, and thus arabinose is probably not released. In contrast, after elongation and differentiation are complete, as in older roots, the cell wall assembly process cleaves the arabinosyl units (6, 13), thus releasing arabinose. This release of arabinose has been observed in isolated coleoptile cell walls (6, 10, 15). Based on this information, we would predict that in a barley seedling that has both seminal roots and adventitious roots, the seminal roots, which are older, would more likely release arabinose than the adventitious roots. Unfortunately, the seminal roots are also closer in proximity to the seedling. Thus, the observation that arabinose-induced JL1157(pAraLHB) cells were frequently on the seminal roots and were rarely observed on the adventitious roots does not support, nor does it argue against, a root origin for the arabinose.

Although the arabinose-responsive biosensor system described here was subject to catabolite repression, it nonetheless responded to the presence of arabinose in both isolated seed exudates and the rhizosphere. Thus, while glucose and other catabolites that can repress P_{BAD} may have been present in the seed, and possibly root, exudates, they evidently were not present in sufficient concentrations to repress the arabinose-induced response. To eliminate this consideration in future studies, the P_{BAD} promoter could be modified to reduce its sensitivity to catabolite repression by removing the region on the promoter where the catabolite activator protein binds (17).

Although the recombination-based biosensor system does not quantify arabinose availability to the biosensor cells, it does indicate the presence or absence of available arabinose at any time during the life history of a cell. This is in contrast to biosensor systems that are designed to quantify availability by relying on transcriptional fusions between a signal-responsive promoter and a reporter gene (2, 16, 18, 20). For such quantitative biosensors, the strength of the reporter signal is a function of the concentration of the inducer; therefore, when the inducer concentration is low, the reporter signal may be weak. In the recombination-based biosensor system described here, the strength of the reporter signal (green fluorescence) was a function exclusively of the expression level of the P_L promoter and was independent of the concentration of inducer present. Thus, a major advantage of this biosensor system over quantitative promoter-reporter fusion-based biosensors is its ability to amplify the reporter signal that results from a low concentration of inducer. In some cases, this amplification function may allow for the detection of a low inducer concentration or a transiently available inducer that would otherwise be missed by a quantitative biosensor.

An important requirement for a recombination-based biosensor is the selection of a signal-responsive promoter that exhibits a low basal level of transcription. As in other recombinase-based genetic systems (4, 5, 21), the production of even low levels of recombinase can lead to a high background level. In this study, leaky transcription from P_{BAD}-*xis,int* led to premature excision of the *c*I cassette, which resulted in a high background level of green fluorescent cells. This high background could be reduced in several ways. First, we have observed that when the *c*I cassette is integrated into the chromosome instead of the plasmid, there is little to no background green fluorescent cells (data not shown). Second, the *att* sequence could be altered to reduce recognition by Xis and Int (4), thus decreasing the sensitivity of the recombinational switch to low concentrations of Xis and Int. However, this could possibly increase the minimal arabinose concentration that the biosensor detects. Third, the translational efficiency of the recombinase genes could be altered (21). Fourth, the high background can be minimized in the cells that are initially deployed into a habitat by using fluorescence-activated cell sorting. In recent studies, we have used this technique to separate the green fluorescent cells, which have resulted from premature excision of the *c*I cassette, from the desired nonfluorescent biosensor cells in an inoculum preparation and have obtained a background of $<1.0\%$ (data not shown).

The design and recombination elements of the site-specific recombination system described here could be applied to the development of a promoter trap to identify genes that are induced transiently, or only to low levels, in vivo (4, 25, 28, 36). A similar system could be used to express a target gene in an environment only under conditions in which that gene product is needed, thus minimizing the metabolic wastefulness that results from expression under nontarget conditions. Such target genes could include genes that kill the cell, thus mediating environmental containment, and genes that confer survival or enhanced competitiveness in a particular microhabitat.

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